Original Article

Viral dynamics of persistent hepatitis C virus infection in high-sensitive reporter cells resemble patient’s viremia

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Abstract  Background: Hepatitis C virus (HCV) infection has a high persistence rate in patients. Although immune cells play a central role in determining the outcomes of HCV infection, the liver is crucial in controlling HCV activity from acute to chronic stages. This investigation grew HCV from a long-term cell culture, and provided an experimental model for studies on HCV persistence in hepatocytes.

Methods: Huh7.5 cells implanted with the NS3/4 protease-based secreted alkaline phosphatase (SEAP) reporter were infected with JFH-1 HCV (moiety of infection = 0.01) and incubated for over 130 days.

Results: The viral activity was obtained by sampling supernatant continuously for SEAP activity measurement. Combined with extracellular and intracellular HCV-RNAs and viral infectivity assays, the experimental results exhibited in vitro viral dynamics resembling the patients’ viremia pattern from acute to chronic infections. The HCV in acute infection comprised exponential accumulation (week 1), plateau (week 2), declining production (weeks 3–4) and silencing (weeks 5–14) phases, and were then reactivated at the onset of chronic infection (after week 15). The HCV-infected cells grew more slowly than the mock controls, and exhibited a
prominent decrease of cell growth rate and increase of early apoptosis in the declining-to-silencing phase transition, suggesting that fitness selection might occur as the infected cells moved across the boundary of active to occult viral activity.

**Conclusion:** Cultivated HCV in the highly sensitive NS3/4-based SEAP reporter cells could establish persistence, which might mimic the viral dynamics from acute to chronic infections in hepatitis C patients.

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**Introduction**

Hepatitis C virus (HCV) persists in 50–80% of infected patients, estimated 185 million persons globally, who are at high risk of liver cirrhosis, hepatocellular carcinoma and metabolic disorders. Current therapies for chronic hepatitis C include interferon-based regimens and the administration of direct antiviral agents. However, treatment of chronic hepatitis C is restricted by the severe side effects and partial effectiveness of interferon, the prohibitively high cost of direct antiviral agents such as Sofosbuvir, and the emergence of resistance strains, and may impose a heavy medical burden in the next two decades. Moreover, a vaccine to prevent HCV infection and disease has not yet been developed. Frequent establishment of HCV chronicity involves highly mutated viral genomes and conquering host immunity. Another possible factor is the viral and cellular changes in hepatocytes, but these have not yet been fully characterized from acute to chronic HCV infections.

HCV, which is a member of the *Flaviviridae* family, has a positive-stranded RNA genome, which encodes three structural and seven non-structural (NS) proteins: in order from the amino- to carboxyl-terminus, the core, envelopes 1 and 2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. Acute HCV infection indicates the first six months of exposure to the virus; and exhibits early phases from RNA detection window to ramp-up, with exponential growth to a plateau. Following the plateau phase, a patient’s plasma HCV-RNA titer may fall, frequently fluctuate by more than 1.0 log, and rebound in chronicity. Close to the end of the HCV viremia plateau, serum alanine aminotransferase reaches its peak level, indicating damage to hepatocytes. Studies have also revealed similar early HCV kinetics and host serum alanine aminotransferase responses in chimpanzees.

As surrogate models, several cultivated cells have been exploited to measure viral genetic evolution and host cell responses that support HCV replicons or HCV infection. Meanwhile, the development of reporter cells has enabled an efficient, real-time and continuous monitor of HCV replication and infection, but has not yet been coupled to building of long-term in vitro cultivation of HCV. The question of whether the viral behaviors caused by taking mere cultivated hepatocytes which were independent of immune cells might reflect patients’ viremia from acute to chronic HCV infections.

In this study, Huh7.5 cells implanted with NS3/4 protease-based secreted alkaline phosphatase (SEAP) reporter were inoculated with a low-dosed HCV (moiety of infection, MOI = 0.01) and analyzed for more than 130 days. Supernatant aliquots were exploited to measure HCV infection, and the viral activities displayed acute (in phases: exponential accumulation, plateau, declining production and silencing) and chronic infections in cultivated hepatocytes, which mimicked those in HCV-infected patients. Cell growth rate, apoptosis, cell cycle, and CD81 expression were measured in parallel. The current NS3/4A-based reporter system is highly sensitive to monitoring HCV activity for a long period, and can be applied to characterize viral and hepatic dynamics at various stages of HCV infection.

**Methods**

**Reporter cells and SEAP activity assay**

Reporter Huh7.5-SEAP cells were established by the integration into Huh-7.5 cells of lentiviral vector comprising the EGFP(Δ4AB)SEAP reporter gene. These cells were grown on a Corning CellBIND Surface 24-well culture plate (Corning, NY, USA), and incubated at 37 °C with 5% CO₂ in Dulbecco’s modified Eagle’s medium that contained 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 1% penicillin/streptomycin (HyClone, South Logan, UT, USA) and 2 g/mL basicidion (Invitrogen, Carlsbad, CA, USA). To avoid over-numbered cell growth constraint, the seeding cell number was 2 × 10⁵ in each well of 24-well plate for initial HCV infection. At post-infection sub-culture every 7 days, each well was seeded with 2 × 10⁶ cells. The SEAP activity in harvested culture media was measured with a Phospha-Light assay kit (Tropix, Foster City, CA, USA).

**Virus purification, determination of virus infectivity and HCV-RNA quantification**

Stable Huh7.5 cells containing the pEF/JFH1-Rz/N plasmid (obtained from Professor T. Jake Liang, NIDDK, Bethesda, MD, USA) were grown to produce HCV virions, which were harvested from the culture medium and concentrated using polyethylene glycol-8000 (Sigma–Aldrich, St. Louis, MO, USA). The HCV infectivity titer was determined by inoculation with Huh7.5 cells and the immunofluorescence staining of HCV core protein, and calculating the number of focus-forming units per milliliter (FFU/mL) on day 3 post-infection. The HCV-RNA titers were quantified using real-time quantitative PCR.
Immunoblot assay and antibodies

Protein samples were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel, and transferred to a polyvinylidene fluoride membrane. Samples were blocked and incubated with the specific primary antibody and then horseradish peroxidase-conjugated secondary antibody. The signal was then developed using Western Blotting (Perkin–Elmer Life Sciences, MA, USA) and quantified using ImageJ software (NIH, Bethesda, MD, USA). The antibodies were mouse monoclonal antibodies against HCV core (C7-50, Abcam, Cambridge, UK), NS3 (8G2, Abcam), NS5A (HCM-131-5, Austral Biological, San Ramon, CA, USA), CD81 (M38, Abcam), and actin (MAB1501, Millipore, Darmstadt, Germany).

Cell viability

One part 0.4% trypan blue and one part cell suspension were incubated at room temperature for 2 min. The unstained living cells were counted using a hemacytometer.

Analysis of cell apoptosis

The harvested cells were incubated for 15 min with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (Annexin Detection kit, Strong Biotech Corporation, Taipei, Taiwan), and detected with FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). The results were analyzed using WinMDI2.9 software (Phoenix, AZ, USA).

Analysis of cell cycle

The cells were fed with Alexafluor® 488-labeled 5′-ethynyl-2′-deoxyuridine (EdU, Click-IT® Plus EdU Flow Cytometry Assay Kit, Invitrogen, CA) for 2 h before harvest, then fixed and permeabilized for 15 min, and treated with RNase A (Zymo Research, Irvine, CA, USA) at 37 °C for 30 min; the DNA contents were stained with 7-aminoactinomycin D (7-AAD, BD Biosciences, San Jose, CA, USA). The fluorescence signals were detected using FACScalibur flow cytometer (BD Biosciences), and analyzed using WinMDI2.9 software (Phoenix, AZ, USA).

Statistical analysis

The comparisons of cells in the early and late apoptosis, and in each phase of the cell cycle between the mock and infected groups were performed using the paired t test. Statistical significance was set to p < 0.05. The statistics were generated in GraphPad Prism version 5.00 software for Windows (www.graphpad.com, San Diego, CA, USA).

Results

Acute and chronic infections of cell-based cultivated HCV

To address the long-term viral activity in vitro, the cell-based cultivation of HCV was characterized in Huh7.5-SEAP reporter cells that had been infected with JFH-1 virus at 0.01 MOI for more than 130 days. In practice, the viral titers without treatment are generally low in chronic hepatitis C patients as compared to those in chronic hepatitis B patients. Therefore, 0.01 MOI was appropriate in our experimental setting. Approximately 50% and 90% of cells were infected at days 6 and 9 post-infection, respectively, as detected by immunostained core protein in our previous studies. Neither the infected cells nor the mock control cells exhibited clear cytopathogenic effects under this experimental condition, and both sets were sub-cultured every seven days in parallel. The measurement of supernatant SEAP activity indicated acute (weeks 1–14) and chronic (after week 15) HCV infections (Fig. 1, top and bottom). The SEAP reporter measurement, which might be translated as viral activity, at the acute stage actively accumulated with an exponential phase (week 1) to reach peak SEAP activity ~120 times that of the mock control, followed by a plateau phase (week 2), a declining production phase (weeks 3–4), then turned into undetectable in a silencing phase (weeks 5–14) and then re-activated by ~30 times that of the mock control at the chronic stage (after week 15) (Fig. 1, top). Approximately 10% of cells were positive for immunostained core protein at the stage of chronic HCV infection. Notably, the duration of each phase varied slightly from the representative experiment in Fig. 1, owing to the use of various batches of virus stock and culture hardware in the repeated experiments. Additionally, the HCV-infected cells, but not the mock control, resisted the secondary infection (Supplementary Fig. 1) in the silencing phase. Furthermore, the expression levels of CD81 protein were downregulated with acute HCV infection and almost undetectable at the chronic stage (Supplementary Fig. 2).

The extracellular viral infectivity (Fig. 2A), extracellular HCV-RNA (Fig. 2B) and intracellular HCV-RNA (Fig. 2C) were measured in parallel on the indicated days, yielding dynamic profiles that reflected the acute and chronic stages similar to those discovered from the SEAP activity in Fig. 1. The peak values in the chronic infection were 1–2 log(s) lower than those in the acute infection (Fig. 2A and C).

With respect to the HCV viral proteins, the results of Western blot analysis (Fig. 3A) revealed that the expressions of core, NS3 and NS5A were significant in the exponential and plateau phases of acute infection, with the quantification results showing that the increments at peak were NS5A > NS3 > core expression in cell lysate preparations (Fig. 3B). Afterwards, the core and NS3 proteins, but not NS5A, were detected at the chronic stage.

Decreased growth rates of the HCV-infected cells

As well as the virus dynamics, the changes of HCV-infected cells were also examined. The cell numbers were counted at the indicated passage time. The mock control cells grew constantly at an average rate of $15.49 \pm 1.41 \times 10^4$ per passage (Fig. 4), while each passage of HCV-infected cells displayed slower growth rates $1.48 \pm 1.04 \times 10^4$ to $15.40 \pm 1.20 \times 10^4$ per passage) than did the mock control cells (Fig. 4). The HCV-infected cells grew at rates ~1/3 of the corresponding mock controls at the exponential and
plateau phases of acute HCV infection. Remarkably, HCV infection caused a strong reduction of the cell growth rate (1.48 ± 1.04 × 10⁴ per passage) at the final passage (week 4) of the declining production phase. The growth rates of the HCV-infected cells then moved upwards steadily and closer to those of the mock control cells until at the chronic stage, when the rates were comparable between the two groups.

**Induction of early apoptosis in the declining-to-silencing phase (D/S) transition of acute HCV infection**

The effect of cell apoptosis or cell cycle arrest on HCV-induced inhibition of the cell proliferation was then tested. The early apoptotic cells had phosphatidylserine externalization and intact cell membrane (annexin V-positive/propidium iodide-negative), and the late apoptotic cells underwent further plasma membrane damage (double positive). The results of apoptosis assay indicate that HCV infection induced a peak of ~60% cells undergoing early apoptosis in the D/S transition (Fig. 5A and B). At the other indicated time points, HCV infection increased the early apoptotic cells by about 10%, as compared to each of the corresponding mock control cells. The late apoptosis was slightly increased in the HCV-infected cells, but was similar to the mock control cells in the D/S transition (Fig. 5A and C). Cell cycle analysis was then undertaken (Supplementary Fig. 3) with the analytical results revealing that the percentages of HCV-infected cells in G1- (Fig. 6A), S- (Fig. 6B) and G2/M (Fig. 6C) phases were comparable to those in the mock control in the D/S transition. This finding suggested that the down-regulation of cell growth and prominent early apoptosis events in the D/S transition are unlikely to be attributable to cell cycle arrest. The percentage of S-
Figure 2. The dynamics of HCV infectivity, extracellular and intracellular HCV-RNA of acute and chronic HCV infections in cultivated hepatocytes. Samples were harvested as presented in Fig. 1. The titers of HCV infectivity (A) and HCV-RNA (B) in supernatant and intracellular HCV-RNA (C) in cell lysate were quantified ($n = 3$). The titers of HCV infectivity at the chronic stage was shown in an enlarged graph inserted in Fig. A. E = exponential phase; P = plateau phase; D = declining production phase; S = silencing phase.
Figure 3. The expression of viral proteins of acute and chronic HCV infections in cultivated hepatocytes. Samples were harvested as presented in Fig. 1. The expression of core, NS3 and NS5A proteins in cell lysate were examined with Western blot analysis (A), and the intensities of bands were quantified (B). E = exponential phase; P = plateau phase; D = declining production phase; S = silencing phase.

Figure 4. The growth rates of HCV-infected and mock control cells. Numbers of living cells were determined at each passage ($n = 3$), and expressed as increment of cells per passage. E = exponential phase; P = plateau phase; D = declining production phase; S = silencing phase.
Figure 5. The early and late apoptosis in HCV-infected and mock control cells. Extent of cell apoptosis was explored with annexin V-FITC and nuclear propidium iodide stains, expressed with dot plots (A) and as percentages of cells in early apoptosis (B) and late apoptosis (C). D/S denotes the week of the transition between declining and silencing phases. Asterisks indicate $p < 0.05$ for the comparison between mock and HCV-infected cells.
phase cells decreased thereafter, but increased in the G2/M phase when entering the silencing phase with acute HCV infection (Fig. 5B and C).

Discussion

Hepatitis C virus (HCV) is prone to establishing chronicity in a natural course of infection. Cell culture has been employed to grow HCV, and provides a feasible and surrogate in vitro method for experimental studies on how hepatocytes might conquer HCV activity. Whereas many studies have been performed using in vitro transient HCV infection for around several days since the robust JFH-1 clone was developed, few have considered long-term cultivation of HCV. Zhong et al. previously reported that HCV and hepatocytes might co-evolve in the development of HCV persistence. Following on from their research, this investigation further characterized the viral dynamics in high-sensitive cultivated reporter cell line, and investigated the interactions between HCV and hepatocytes. Identifying cell-based persistent HCV infections is very important to support amendable studies bearing resemblance to patients' chronic hepatitis C.

Current in vitro reporter assays are extensively applied for screening of direct anti-HCV compounds. The analytical platforms measure efficient and constant detectable targets by either direct fusing a reporter to HCV genome, or genetically engineering a reporter into each host cell separately. The reporters comprise of fluorescent or enzymatic tags, such as luciferase, fluorescence resonance energy transfer substrate, chloramphenicol transferase and SEAP. However, long-term cell culture with HCV infection has been neither performed by continuous monitor of high sensitive reporters, nor applied to experiments aimed at delineating HCV pathogenesis. The following conclusions were drawn from the high sensitivity NS3/4A-based SEAP reporter adopted in this study: (i) the NS3 was superior to core and NS5A, because the NS3 protein stably accumulated intracellularly with HCV infection at both acute and chronic stages (Fig. 3), and (ii) the supernatant harvests were continuously analyzed without lysis of the cells. Furthermore, the SEAP reporter was not constructed from the HCV genome that might potential attenuate viral replication and production. Experimental results from the current high-sensitivity NS3/4A-based SEAP reporter exhibited an in vitro viral dynamic that resembled the patients' viremia pattern from acute to chronic HCV infections. Because the present cultivated reporter platform had no interference from other cells, such as immune cells, the viral activities might be a genuine reflection of mere interactions between the hepatocytes and the virus.

Figure 6. The cell cycle analysis in HCV-infected and mock control cells. Cell cycle was explored with EdU-Alexa 488 and 7-AAD stains and expressed as percentages of cells in G1 (A), S (B) and G2/M (C) phases. D/S denotes the week of the transition between declining and silencing phases. Asterisks indicate p < 0.05 for the comparison between mock and HCV-infected cells.
Nevertheless, the current NS3/4A based SEAP reporter system can be utilized to monitor HCV activity over a long period of persistent HCV infection.

The host defenses to control viral infections include immune and hepatic responses, both of which seem to be overcome by HCV, enabling this viral infection to persist in the majority of the infected patients. In this study, the HCV activity in cultivated hepatocytes revealed similar dynamics to patients’ viremia from an exposure to the virus by HCV-RNA quantification that undergoes exponentially rises to plateau, declines, silences and rebounds to establish chronicity. Therefore, current experimental results indicate that the hepatic response was activated to control HCV infection. However, even in the silencing phase, HCV were not completely eradicated although the viral RNA and proteins were below detection limits, and HCV chronicity developed subsequently. The HCV-infected cells in the silencing phase were found to be refractory to the secondary HCV infection (Supplementary Fig. 1), further indicating that the anti-HCV defense might function appropriately in the hepatocytes. The later reactivation of HCV at the chronic stage reflected a failure of the hepatic defense where the mechanism remains elusive. In addition, down-regulation of surface co-receptor CD81 represents a hepatic anti-HCV defense mechanism, as reported by Zhong et al. previously and in this study. CD81 might also participate in HCV-RNA replication, which conversely reduced CD81 total and surface expressions by intracellular retain. Experimental results further indicate that the CD81 protein expression was reduced as early as in the exponential phase and almost undetectable thereafter, even in the declining and silencing phases when the viral activity was occult, suggesting that active HCV-RNA replication might not be the only cause of hepatic CD81 reduction.

The vigorous HCV activities retarded the cell growth rate (Fig. 4) at the exponential and plateau phases of acute infection. This phenomenon might indicate that the virus has taken over host general machinery for its own propagation. However, as in the declining and silencing phases, the number of HCV-infected cells still multiplied more slowly than did the mock control cells (Fig. 4), indicating that the cell growth rate was affected with HCV infection by the other mechanism. As shown at the chronic stage (Fig. 4), the HCV-infected cells were adapted to grow constantly at comparable rates to their mock counterparts in the context of supporting mild HCV production.

Apoptosis is a host anti-viral defense to destroy infected cells. Previous experimental studies have demonstrated that HCV infection and HCV proteins might promote or inhibit apoptosis, indicating a subtle imbalance between pro- and anti-apoptotic potentials in the HCV-infected cells. Nevertheless, chronic hepatitis C patients exhibited enhanced apoptosis in liver from 0.54% to 20.00% of hepatocytes, which might be correlated with liver pathogenesis and fibrogenesis. Consistently with previous investigations, the proportions of apoptotic cells were higher in the HCV-infected group than in the corresponding mock control in both the acute and chronic stages in this study (Fig. 5). Notably, prominent early cell apoptosis occurred in the D/S transition. The early apoptosis was characterized as annexin V-positive/propidium iodide-negative, implying that the cells had surface exposure of phosphatidylserine moiety, without permeability of the plasma membrane. This atypical apoptosis pattern was not documented before with HCV-infected hepatocytes that exhibited annexin V-positive/propidium iodide-positive in late apoptosis, but has been reported with HCV-infected pancreatic beta cells. Wang et al. inoculated HCV virions to infect the insulinoma cell line, MIN6, where HCV could replicate at a low efficiency but not generate infectious progeny viruses. The HCV-induced apoptosis in the MIN6 cells were revealed as annexin V-positive/propidium iodide-negative, similar to the immunophenotyping profile of the hepatocytes in the D/S transition in the current study (Fig. 5A, D/S time point). The HCV-induced apoptosis of MIN6 cells involved endoplasmic reticulum stress, mitochondria impairment and activation of caspase 3, and led to nuclear DNA fragmentation. Moreover, the apoptotic MIN6 cells exhibited a dilated, low-density and membrane-intact nucleus that was distinct from the canonical chromatin condensed morphology of late apoptotic cells characterized as annexin V and propidium iodide double positive. Significantly, the HCV-induced early apoptosis in hepatocytes did not depend on high efficiency of HCV infectivity, but could be induced by prolonged stresses through a mechanism remaining elusive. This finding has two implications: (i) reduction of HCV activity at the end of the declining phase might induce early apoptosis of the infected cells, and (ii) the early apoptosis events might lower HCV activity, than the onset of silencing phase occurred. Moreover, Geske et al. indicated that p53-induced early apoptosis in mammary epithelial cells is reversible through the nucleotide excision DNA repair function. Their finding implies that the rescued early apoptotic, infected hepatocytes may contribute to HCV persistency or oncogenesis. Nevertheless, the hepatic early apoptosis suggests that fitness selections may occur as the infected cells move across the boundary of supporting to non-supporting active viral activity, thus possibly establishing HCV persistence.

In conclusion, long-term cultivation of HCV in NS3/4A protease-based reporter cells was conducted to mimic viremia in patients with acute to chronic HCV infections. The developed in vitro model can be adopted to elucidate HCV pathogenesis at various stages of HCV infection in hepatocytes.

Conflicts of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jmii.2016.11.004.